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Capability of 11 Antipneumococcal Antibiotics To Select for Resistance by Multistep and Single-Step Methodologies▽†

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Testing of 12 pneumococcal strains with differing resistotypes [including *tet*(M) positive] showed that tigecycline, amoxicillin-clavulanate, imipenem, and ceftriaxone did not select for resistant clones after 50 sequential subcultures. By comparison, azithromycin, clarithromycin, clindamycin, telithromycin, levofloxacin, moxifloxacin, and gemifloxacin did show resistant clones. Tigecycline also yielded a low frequency of resistance in single-step tests compared to all β-lactams, macrolides/ketolides, and quinolones tested.

The incidence of pneumococci resistant to penicillin G and other β-lactam and non-β-lactam compounds is increasing worldwide. Penicillin and macrolide resistance rates of at least 50% have been observed in many countries, and the major foci of resistance currently include Spain, France, Central and Eastern Europe, and Asia (8, 11). Jacobs and coworkers (8) have reported the worldwide prevalence of pneumococci with MICs of >2.0 μg/ml isolated during 1998 and 2000 to be 18.2%, with an overall macrolide resistance rate of 24.6%. Coresistance between penicillin G and doxycycline in the latter study was 61.2%.

Although the introduction of a pediatric conjugate vaccine has led to a dramatic decrease in systemic infections (e.g., meningitis and bacteremia) caused by drug-resistant pneumococci, this dramatic decrease has not been mirrored in pneumococcal community-acquired respiratory tract infections caused by resistant strains, which still occur, albeit at lower rates than before the pediatric vaccine era (especially in cases of otitis media). Also, nonvaccine serotypes with raised penicillin G MICs have recently appeared (10). There is thus still a need for new agents to treat these infections.

Tigecycline is a broad-spectrum glycylcycline, which has recently been approved in the United States for use in complicated skin and soft tissue infections and complicated intra-abdominal infections. This compound has a very broad in vitro susceptibility spectrum, including against *Streptococcus pneumoniae* (1, 2, 6). An FDA indication for tigecycline in the treatment of community-acquired pneumonia is currently being pursued by the manufacturer.

This study attempted to broaden the information on the in vitro antipneumococcal activity of tigecycline by using multistep and single-step methodologies to test the abilities of tigecycline, amoxicillin/clavulanate, ceftriaxone, imipenem, azithromycin, clarithromycin, telithromycin, clindamycin, levofloxacin, moxifloxacin, and gemifloxacin to select for resistant mutants in 12 pneumococcal strains. Comparator drugs were selected to reflect currently available and commonly used therapeutic modalities for the oral and intravenous treatment of community-acquired pneumonia.

The 12 pneumococcal strains comprised 4 each of penicillin G-susceptible, -intermediate, and -resistant pneumococci from clinical isolates. Of these, four strains were macrolide susceptible [one strain had *erm*(B)], eight were macrolide resistant [two strains were *erm*(B), two *mef*(A), one *erm*(B) *mef*(A), and one *erm*(A), and there was one strain with 23S rRNA and one with L4 mutations], and three were quinolone resistant with defined mutations in the quinolone resistance-determining region. Strain 2527 had *erm*(B) as detected by PCR, but remained susceptible to all macrolides tested. This phenomenon may be explained by the fact that the *erm*(B) gene was not expressed in this strain. Five strains were resistant and one was intermediate to tetracycline, and all carried *tet*(M) on three different transposons (Table 1). Tigecycline was obtained from Wyeth Laboratories, Collegeville, MD. Other antimicrobials were obtained from their respective manufacturers.

Serial passages were performed daily from each strain in subinhibitory concentrations of all antimicrobials (5, 12). For tigecycline testing, fresh broth medium was used in order to standardize the methodology (1). In all cases, the broth medium was 1 ml per tube of cation-adjusted Mueller-Hinton broth (BBL) with 5% lysed horse blood. For each subsequent daily passage, an inoculum (10 μl) of one to two dilutions below the MIC that matched the turbidity of a growth control tube was taken from the tube. The above inoculum was used to determine the next MIC. Daily passages were performed until a significant increase in MIC (≥8 times) was obtained. A minimum number of 14 passages was performed in every case. The maximal number of passages was 50. The stability of the acquired resistance was determined by determining the MIC after 10 daily passages of the mutant on blood agar without antibiotics (5, 12). The MICs for each resistant pneumococcal clone of each compound were determined from the macrodilution MIC (4). The identities of the obtained mutants and their respective parents were confirmed by pulsed-field gel electrophoresis at the end of the study (10). Pulsed-field gel electrophoresis of SmaI-digested DNA was performed using a CHEF DR III apparatus (Bio-Rad, Hercules, CA) with the following run parameters: a switch time of 5 to 20 s and a run

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TABLE 1. Resistance phenotypes and mechanisms of the 12 pneumococcal strains studied[a]

Strain	Phenotype for:[c]				Resistance mechanism(s) against:[b]		
	PEN	ERY	TET	LVX	ERY	TET	LVX
3009	S	R	R	S	23S rRNA (A2058G)	*tet*(M), Tn*916*-like	NT
1076	S	R	R	I	*erm*(B) *mef*(A)	*tet*(M), Tn*5253*-like	GyrA (S81Y), ParC (D83N)
1077	S	S	S	R	NT	NT	GyrA (S81Y), ParC (S79F), ParE (I460V)
1635	S	R	S	S	*erm*(A)	NT	NT
3665	I	R	S	S	*mef*(A)	NT	NT
3676	I	R	S	S	*mef*(A)	NT	NT
3243	I	S	R	S	−	*tet*(M), Tn*5253*-like	NT
3274	I	S	S	S	NT	NT	NT
24	R	R	R	S	*erm*(B)	*tet*(M), Tn*916*-like	NT
37	R	R	I	S	*erm*(B)	*tet*(M), Tn*916*-like	NT
3413	R	R	S	S	Altered L4	NT	NT
2527	R	S	R	R	*erm*(B)	*tet*(M), Tn*916*-like, Tn*5252*-like	GyrA (S81Y), ParC (S79F, K137N), ParE (I460V)

[a] PEN, penicillin; ERY, erythromycin; TET, tetracycline; LVX, levofloxacin; NT, not tested; I, intermediate; R, resistant; S, susceptible; −, *erm*(B) and *mef*(A) not detected.
[b] Tested among nonsusceptible strains.
[c] MIC interpretive standards (μg/ml) are as follows. PEN: S, <0.06; I, 0.125 to 1; R, >2. ERY: S, <0.25; I, 0.5; R, >1. TET: S, <2; I, 4; R, >8. LVX: S, <2; I, 4; R, >8.

time of 16 h (5, 12). Selected resistant clones were examined for changes in resistance mechanisms as described below.

The frequency of spontaneous single-step mutations was determined by spreading suspensions (approximately 10^{10} CFU/ml) on Mueller Hinton agar (BBL) with 5% sheep blood at 2, 4, and 8 times the MIC (12). After incubation at 35°C in 5% CO_2 for 48 h, the resistance frequency was calculated as the number of colonies with MICs that were at least four times greater than the parental MIC per inoculum. Single-step studies were not performed with azithromycin, clarithromycin, clindamycin, and levofloxacin for strains with MICs of ≥16 μg/ml.

All macrolide- and tetracycline-nonsusceptible clinical strains and -resistant clones (Table 1) were tested for the presence of *erm*(B), *erm*(A), and *mef*(A) genes by PCR amplification. The presence of mutations in L4 and L22 proteins and 23S rRNA was examined by using primers and conditions described previously (3, 5, 13). Nucleotide sequences were obtained by direct sequencing using a CEQ8000 genetic analysis system (Beckman Coulter, Fullerton, CA). Quinolone-resistant strains were tested for mutations in type II topoisomerase as described previously (12). All tetracycline-nonsusceptible strains were tested for the presence of *tet*(M) and *tet*(O), and the identification of transposons was done as described previously (7).

The results of the multistep studies are summarized in Table 2, which also summarizes the resistotype of each strain studied. As can be seen, the MIC (μg/ml) ranges for the parent strains were as follows: for tigecycline, 0.016 to 0.03; for amoxicillin-clavulanate, 0.016 to 2; for ceftriaxone, 0.016 to 2; for imipenem, 0.002 to 0.5; for azithromycin, 0.008 to >64; for clarithromycin, 0.008 to >64; for telithromycin, 0.004 to >64; for clindamycin, 0.008 to >64; for levofloxacin, 0.5 to 16; for moxifloxacin, 0.125 to 4; and for gemifloxacin, 0.016 to 0.25. After 50 subcultures, tigecycline did not yield resistant mutants from any strains tested [including *tet*(M) strains], and the MICs were in the same range as for parent strains. All three β-lactams also did not yield resistant clones in the 12 strains tested after 50 days. Of seven strains tested, azithromycin yielded resistant mutants in four strains (0.016 to 2 μg/ml [parental MIC range] increased to 0.25 to >64 μg/ml [mutant MIC ragne]; 14 to 33 days). Clarithromycin had resistant clones in 5 of 9 strains tested (0.016 to 16 μg/ml [parental MIC range] increased to 0.25 to >64 μg/ml [mutant MIC ragne]; 25 to 49 days), and telithromycin had resistant clones in 9 of 12 strains tested (0.004 to 0.5 μg/ml [parental MIC range] increased to 0.06 to >64 μg/ml [mutant MIC range]; 14 to 48 days). Two of nine strains tested with clindamycin had resistant clones (0.016 to 0.06 μg/ml [parental MIC range] increased to 4 to >64 μg/ml [mutant MIC range]; 14 to 49 days). One of 12 strains with levofloxacin (1 μg/ml [parent MIC] increased to 16 μg/ml [mutant MIC]; 18 days), 2 of 12 with gemifloxacin (0.016 to 0.25 μg/ml [parental MIC range] increased to 1 to 2 μg/ml [mutant MIC range]; 14 to 21 days), and 6 of 12 with moxifloxacin had resistant clones (0.125 to 1 μg/ml [parental MIC range] increased to 1 to 8 μg/ml [mutant MIC range]; 19 to 50 days).

Out of 20 macrolide-, telithromycin-, and clindamycin-resistant mutants, 11 developed mutations in 23S rRNA (A2058G, A2059T/C, and A2106G) and ribosomal proteins L22 (insertions 93SFRPRA94 and 92RVRP93) and L4 (G69R). However, in 9 macrolide-, telithromycin-, and clindamycin-resistant mutants, no alterations were found in the studied regions of 23S rRNA and ribosomal proteins L4 and L22. Among nine mutants selected with quinolones, all but one moxifloxacin-resistant mutant had mutations in GyrA (S81Y/F and E85Q/G), ParC (S79Y/F and D83N), and GyrB (P413S and S478I).

Single-step analysis (see Table S3 in the supplemental material) showed mutation frequencies as follows: for tigecycline, $<1.7 \times 10^{-10}$ to $<6.7 \times 10^{-9}$ at both 2× MIC and 8× MIC; for amoxicillin/clavulanate, $<5.0 \times 10^{-11}$ to $<4.5 \times 10^{-10}$ at both 2× and 8× MIC; for imipenem, $<1.1 \times 10^{-10}$ to $<2.2 \times 10^{-9}$ at both 2× and 8× MIC; for ceftriaxone, $<1.3 \times 10^{-10}$ to $<2.0 \times 10^{-9}$ at both 2× and 8× MIC; for azithromycin, 2.8×10^{-9} to 8.0×10^{-6} at 2× MIC and $<1.9 \times 10^{-10}$ to 4.0×10^{-7} at 8× MIC; for clarithromycin, $< 5.0 \times 10^{-10}$ to 9.8×10^{-9} at 2× MIC and $<1.2 \times 10^{-10}$ to 3.5×10^{-9} at 8× MIC; for telithromycin, $< 1.0 \times 10^{-9}$ to 1.3×10^{-4} at 2× MIC and

TABLE 2. *Streptococcus pneumoniae* multistep resistance selection results[a]

Strain	Antibiotic	Initial MIC	MIC after resistance selection (no. of passages)	MIC after 10 antibiotic-free subcultures[c]:											Resistance mechanism(s) of mutants
				TGC	AMC	TEL	AZM	CLI	CLR	IPM	CRO	GEM	LVX	MXF	
3009	TGC	0.03	0.016 (50)												
	AMC	0.016	0.03 (50)												
	TEL	0.016	0.03 (50)												23S rRNA (A2058G)[b]
	AZM	>64	NT												23S rRNA (A2058G)[b]
	CLI	1	2 (50)												
	CLR	16	>64 (25)	0.03	≤0.008	0.06	>64	1	64	≤0.004	0.016	0.03	0.5	0.125	23S rRNA (A2058G)[b]
	IPM	0.008	0.008 (50)												
	CRO	0.03	0.03 (50)												
	GEM	0.016	1 (21)	0.016	0.008	0.03	>64	0.5	16	0.008	0.03	2	**8**	**2**	GyrA (E85Q), ParC (D83N)
	LVX	1	16 (18)	0.06	≤0.008	0.016	>64	0.5	8	≤0.004	0.016	**1**	32	**4**	GyrA (S81Y), ParC (S79Y)
	MXF	0.25	2 (19)	0.06	0.03	0.008	>64	0.5	8	0.008	0.03	**0.125**	2	4	GyrA (S81F), ParC (D83N)
1076	TGC	0.03	0.03 (50)												
	AMC	0.016	0.03 (50)												
	TEL	0.5	>64 (14)	0.03	0.016	>64	>64	>64	>64	0.004	0.03	0.25	8	1	Not found
	AZM	>64	NT												
	CLI	64	NT												
	CLR	>64	NT												
	IPM	0.008	0.008 (50)												
	CRO	0.016	0.016 (50)												
	GEM	0.25	0.5 (50)												
	LVX	4	16 (50)												
	MXF	1	8 (35)	0.06	0.03	1	>64	>64	>64	0.008	0.016	0.5	**32**	4	ParC (S79Y)
1077	TGC	0.016	0.016 (50)												
	AMC	0.016	0.03 (50)												
	TEL	0.004	0.25 (15)	0.03	0.016	0.25	**>64**	**8**	**>64**	0.008	0.016	0.25	16	4	23S rRNA (A2058G, A2106G)
	AZM	0.03	>64 (29)	0.03	0.03	**0.125**	>64	**4**	**>64**	0.008	0.03	0.25	32	4	23S rRNA (A2058G)
	CLI	0.016	4 (49)	0.03	0.016	**0.06**	**>64**	8	**>64**	0.004	0.016	0.25	16	4	23S rRNA (A2058G, A2106G)
	CLR	0.016	0.008 (50)												
	IPM	0.008	0.008 (50)												
	CRO	0.016	0.016 (50)												
	GEM	0.25	1 (50)												
	LVX	16	32 (50)												
	MXF	4	4 (50)												
3665	TGC	0.016	0.016 (50)												
	AMC	0.125	0.5 (50)												
	TEL	0.125	2 (14)	0.03	0.06	0.25	4	0.03	2	0.008	0.06	0.03	0.5	0.125	Not found
	AZM	8	16 (50)												
	CLI	0.016	0.03 (50)												
	CLR	2	16 (26)	0.03	0.25	0.06	8	0.03	8	0.03	0.03	0.03	0.5	0.06	Not found
	IPM	0.03	0.016 (50)												
	CRO	0.06	0.06 (50)												
	GEM	0.016	0.06 (50)												
	LVX	0.5	2 (50)												
	MXF	0.125	1 (38)	0.03	0.25	0.125	8	0.03	2	0.03	0.06	**0.125**	**4**	1	GyrA (S81Y)
3676	TGC	0.016	0.016 (50)												
	AMC	0.03	0.125 (50)												
	TEL	0.06	2 (14)	0.03	0.03	0.06	2	0.03	2	0.008	0.06	0.06	1	0.5	Not found
	AZM	4	8 (50)												
	CLI	0.03	0.016 (50)												

	CLR	4	32 (44)	0.03	0.016	0.03	16	0.06	16	0.016	0.03	0.03	1	0.25	Not found
	IPM	0.016	0.016 (50)												
	CRO	0.03	0.06 (50)												
	GEM	0.016	0.03 (50)												
	LVX	0.5	2 (50)												
	MXF	0.25	8 (50)	0.03	0.06	0.06	4	0.016	4	0.03	0.03	**1**	**16**	8	GyrA (E85G), GyrB (S478I), ParC (S79Y)
3243	TGC	0.03	0.03 (50)												
	AMC	0.06	0.25 (50)												
	TEL	0.004	0.06 (29)	0.03	0.125	0.06	**>64**	0.03	**>64**	0.016	0.125	0.03	1	0.25	Not found
	AZM	0.03	0.25 (33)	0.06	0.125	**0.06**	0.5	0.016	**0.5**	0.03	0.125	0.03	0.5	0.25	L22 (insertion 93SFRPRA94)
	CLI	0.016	0.03 (50)												
	CLR	0.016	0.25 (41)	0.03	0.06	0.016	**1**	**1**	0.25	0.03	0.125	0.03	0.5	0.125	23S rRNA (A2059T)
	IPM	0.016	0.016 (50)												
	CRO	0.125	0.25 (50)												
	GEM	0.016	0.06 (50)												
	LVX	0.5	2 (50)												
	MXF	0.125	1 (20)	0.03	0.06	0.016	0.016	0.016	0.004	0.004	0.06	0.06	1	1	Not found
1635	TGC	0.016	0.03 (50)												
	AMC	0.016	0.016 (50)												
	TEL	0.004	0.008 (50)												
	AZM	2	>64 (14)	0.03	0.008	0.004	>64	0.03	**>64**	0.002	0.016	0.03	1	0.25	Not found
	CLI	0.06	>64 (14)	0.03	0.008	0.008	4	>64	0.5	0.002	0.016	0.03	1	0.5	Not found
	CLR	0.5	>64 (49)	0.016	0.008	0.016	**>64**	0.25	>64	0.002	0.016	0.03	1	0.25	23S rRNA (A2059C)
	IPM	0.002	0.002 (50)												
	CRO	0.016	0.03 (50)												
	GEM	0.016	0.06 (50)												
	LVX	1	1 (50)												
	MXF	0.25	0.25 (50)												
24	TGC	0.03	0.03 (50)												
	AMC	1	2 (50)												
	TEL	0.25	32 (14)	0.03	2	16	>64	>64	>64	0.25	2	0.016	1	0.125	L4 (G69R)
	AZM	>64	NT												
	CLI	>64	NT												
	CLR	>64	NT												
	IPM	0.5	0.5 (50)												
	CRO	1	2 (50)												
	GEM	0.016	0.03 (50)												
	LVX	1	2 (50)												
	MXF	0.25	4 (50)	0.03	1	0.25	>64	>64	>64	0.5	1	**0.5**	**32**	4	GyrA (S81Y), GyrB (P413S), ParC (S79F)
2527	TGC	0.016	0.016 (50)												
	AMC	1	2 (50)												
	TEL	0.004	0.016 (50)												
	AZM	0.016	4 (20)	0.03	2	**0.03**	4	**0.06**	**16**	0.5	2	0.25	32	4	Not found
	CLI	0.008	0.016 (50)												
	CLR	0.008	0.016 (50)												
	IPM	0.25	0.25 (50)												
	CRO	2	8 (50)												
	GEM	0.25	2 (14)	0.03	0.5	0.004	0.03	0.016	0.016	0.125	1	2	32	8	GyrA (S81YS,[b] E85K), ParC (S79F)[b]
	LVX	16	32 (50)												
	MXF	2	8 (50)												

Continued on following page

TABLE 2—*Continued*

Strain	Antibiotic	Initial MIC	MIC after resistance selection (no. of passages)	MIC after 10 antibiotic-free subcultures[c]:											Resistance mechanism(s) of mutants
				TGC	AMC	TEL	AZM	CLI	CLR	IPM	CRO	GEM	LVX	MXF	
3274	TGC	0.03	0.03 (50)												
	AMC	0.125	0.25 (50)												
	TEL	0.004	0.5 (48)	0.03	0.25	0.5	**1**	0.016	**1**	0.016	0.125	0.03	1	0.125	L22 (insertion 92RVRP93)
	AZM	0.008	0.008 (50)												
	CLI	0.016	0.008 (50)												
	CLR	0.008	0.03 (50)												
	IPM	0.016	0.03 (50)												
	CRO	0.125	0.25 (50)												
	GEM	0.016	0.06 (50)												
	LVX	0.5	0.5 (50)												
	MXF	0.125	0.25 (50)												
37	TGC	0.016	0.016 (50)												
	AMC	2	2 (50)												
	TEL	0.25	4 (14)	0.03	2	8	>64	>64	>64	0.25	2	0.03	1	0.25	Not found
	AZM	>64	NT												
	CLI	>64	NT												
	CLR	>64	NT												
	IPM	0.5	0.5 (50)												
	CRO	2	1 (50)												
	GEM	0.016	0.016 (50)												
	LVX	1	1 (50)												
	MXF	0.125	0.25 (50)												
3413	TGC	0.016	0.03 (50)												
	AMC	2	8 (50)												
	TEL	0.03	0.25 (30)	0.03	4	0.5	>64	0.06	16	0.125	2	0.03	0.5	0.25	Not found
	AZM	>64	NT												
	CLI	0.016	0.008 (50)												
	CLR	8	8 (50)												
	IPM	0.125	0.06 (50)												
	CRO	1	1 (50)												
	GEM	0.016	0.016 (50)												
	LVX	0.5	1 (50)												
	MXF	0.125	0.25 (50)												

[a] All MIC values are in μg/ml. TGC, tigecycline; AMC, amoxicillin-clavulanate; TEL, telithromycin; AZM, azithromycin; CLI, clindamycin; CLR, clarithromycin; IPM, imipenem; CRO, ceftriaxone; GEM, gemifloxacin; LVX, levofloxacin; MXF, moxifloxacin; NT, not tested. Cross reactivity is denoted by bold font.

[b] Mutation was present in the parent strain.

[c] Determinations of MICs after 10 antibiotic-free subcultures were not performed for strains for which the MIC change from the parental MIC after 50 days was ≤4-fold.

$<1.5 \times 10^{-10}$ to 4.8×10^{-6} at 8× MIC; for clindamycin, $<1.8 \times 10^{-10}$ to 1.7×10^{-4} at 2× MIC and $<1.2 \times 10^{-10}$ to 5.6×10^{-7}at 8× MIC; for levofloxacin, $<1.0 \times 10^{-10}$ to 7.9×10^{-8} at 2× MIC and $<1.0 \times 10^{-10}$ to $<3.7 \times 10^{-10}$ at 8× MIC; for moxifloxacin, $<1.0 \times 10^{-10}$ to 8.3×10^{-8} at 2× MIC and $<1.0 \times 10^{-10}$ to $<1.0 \times 10^{-8}$ at 8× MIC; and for gemifloxacin, $< 1.1 \times 10^{-10}$ to 1.3×10^{-4} at 2× MIC and $<1.1 \times 10^{-10}$ to $<6.3 \times 10^{-10}$ at 8× MIC. In the current study, tigecycline yielded uniformly low MICs against all strains tested and did not yield resistant mutants in multistep studies even after 50 subcultures. An efflux pump similar to that described by McAleese and coworkers for *Staphylococcus aureus* (9) was not found. Tigecycline also gave very low resistance rates in single-step studies in both *tet*(M)-positive and -negative strains. The lack of resistance selection by β-lactams but selection of resistant clones by macrolides, ketolides, and quinolones in pneumococci confirms previous findings by our group (5, 12).

The low MICs obtained for tigecycline, as well as the lack of resistance selection after 50 days and low resistance frequency in single-step studies against both *tet*(M)-positive and *tet*(M)-negative pneumococci, point to a very useful future for tigecycline in the treatment of pneumococcal community-acquired pneumonia.

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